



OPEN First report on isolation and characterization of *Bacillus* Sp. associated with honey bee brood disease

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Bacterial infections in honey bee brood pose a significant threat to bee populations, leading to decreased honey production and disrupting critical crop pollination networks. While *Paenibacillus* larvae and *Melissococcus plutonius* are well-established as major pathogens of honey bee eggs and larvae, the presence of other harmful bacteria in the Khyber Pakhtunkhwa (KPK) province of Pakistan remains largely unexplored. Molecular characterization of *Bacillus* species and *B. cereus* isolates revealed the presence of key virulence genes, including the *cry* gene, known for its insecticidal properties. The ability of *B. cereus* to produce cytotoxins, haemolysins, and enterotoxins raises concerns about its impact on larval immunity and survival. Similarly, *B. mycoides*, a close relative of *B. cereus*, was identified in diseased broods, highlighting its potential involvement in microbial dysbiosis within the hive. Reports from North America and Europe have also linked these *Bacillus* species to declining honey bee health, emphasizing the need for further investigation into their pathogenic mechanisms. This study investigated bacterial contamination in broods collected from infected beehives across various cities of KPK. Biochemical and molecular analyses revealed a widespread presence of bacteria the *Bacillus* species, as the emerging most dominant, followed by *Bacillus cereus*. Phylogenetic analysis indicated a close evolutionary relationship between *Bacillus* species, and *Bacillus cereus*, highlighting their shared spore-forming characteristics. This research is the first to report the involvement of *Bacillus* species in infecting honey bee eggs and larvae, shedding light on a previously unrecognized threat to apiculture and pollination in the region.

Keywords Honey bee, *Paenibacillus* larvae, *Bacillus* species, *Bacillus cereus*, Pollination, Phylogenetic analysis

Honey, a natural product highly valued for its nutritional and medicinal properties, exhibits remarkable longevity and resistance to spoilage due to its low water activity and high sugar concentration¹. Despite these antimicrobial attributes, certain resilient microbial species can persist in this environment, posing a serious threat to honey bee health and overall colony sustainability². Microbial contamination in honey typically originates from environmental sources such as pollen, flowers, air, dust, and the honey bee's digestive system, with yeasts and bacteria forming the predominant microflora within beehives^{3,4}. While honey bee larvae hatch in a sterile state, they are rapidly exposed to microbes through interactions with worker bees, pollen, and hive surroundings. The maintenance of healthy broods is crucial, as they replenish the short-lived worker bee population, sustain honey production, and ensure effective crop pollination^{5,6}. However, honey bee populations worldwide are increasingly threatened by microbial pathogens, including viruses, fungi, and bacteria⁷.

Pakistan is home to diverse honey bee species, including the native *Apis cerana* and the introduced *Apis mellifera*, both of which play a vital role in honey production and crop pollination. Beekeeping is an important agricultural practice in the country, with approximately 10,000 registered beekeepers managing over 1.5 million

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colonies, producing more than 12,000 metric tons of honey annually⁸. The honey produced in Pakistan, particularly the Sidr (*Ziziphus*) honey, is renowned for its high quality and medicinal value, contributing significantly to the local economy and exports. Beekeeping not only provides livelihoods to thousands of families but also enhances agricultural productivity through pollination services, benefiting crops such as mustard, sunflower, and various fruits^{8,9}.

Despite its economic and ecological significance, honey bee populations in Pakistan face multiple threats, including habitat destruction, climate change, pesticide exposure, and pathogen infections⁹. Brood diseases, in particular, pose a substantial risk to honey bee health, with bacterial pathogens contributing to colony losses. Among these, *Paenibacillus larvae* and *Melissococcus plutonius*—the causative agents of American foulbrood (AFB) and European foulbrood (EFB), respectively—are the most well-documented brood diseases worldwide, causing significant mortality in honey bee colonies^{10,11}.

AFB, caused by the spore-forming *P. larvae*, is one of the most devastating bacterial diseases affecting honey bee broods. If left untreated, it can rapidly decimate entire colonies, as its highly resilient endospores allow the pathogen to persist in hives for years^{12,13}. In contrast, EFB, caused by the non-spore-forming *M. plutonius*, primarily affects young larvae by outcompeting them for nutrients, leading to starvation and eventual death in the early developmental stages^{14,15}.

Beyond these well-documented brood pathogens, the hive microbiome also harbors other spore-forming bacteria, including *Bacillus cereus*, a member of the *Bacillus* group, which comprises closely related species such as *Bacillus mycoides*^{16,17}. While *B. cereus* is widely recognized for its pathogenic potential in humans, its impact on honey bee health remains unexplored. Given the close phylogenetic relationship between *B. cereus*, *Paenibacillus larvae*, and other *Bacillus* species, the potential role of *Bacillus* spp. in honey bee brood disease warrants further investigation¹⁸.

The increasing occurrence of unexplained colony losses highlights the urgent need to investigate honey bee brood diseases beyond well-known pathogens^{19,20}. However, emerging pathogens in the hive microbiome remain poorly studied in Pakistan, necessitating further investigation. This study aims to molecularly screen honey bee samples to assess the presence and diversity of *Bacillus* species-associated bacterial pathogens in the Khyber Pakhtunkhwa region of Pakistan. The findings contribute to a broader understanding of brood diseases and lay the groundwork for future studies aimed at protecting honey bee populations in the region.

Materials and methods

Study area

The samples were collected from ten different beehives containing diseased broods, found in the selected districts of central KPK provinces comprising of Mardan (34.1986° N, 72.0404° E), Nowshera (34.0105° N, 71.9876° E), Charsadda (34.1527° N, 71.7468° E), Swabi (34.1270° N, 72.4741° E), and Peshawar (34.0151° N, 71.5249° E). A Piece of about 20 cm² containing a maximum portion of dead and discolored brood was obtained from each beehive. In this study geographical coordinates were identified using a Global Positioning System GPS. These coordinates were further used in the R Statistical Software version 4.3.1 to generate distribution map of the analyzed regions as the one shown in Fig. 1.

Honey bee collection and identification

All brood samples analyzed in this study originated from symptomatic colonies. The climate of these selected regions varied with temperature reaching as low as 5 °C in January to as high as 40 °C in June. A total of 460 honey bee (including larvae and eggs) was collected from the five districts during January 2023 to June 2024. These larvae's and eggs were randomly selected despite symptomatic or asymptomatic nature of the larvae. A total of 185 specimens were collected from District Mardan, followed by 121 specimens from Peshawar, 92 specimens from Nowshera, and finally 62 from the district Swabi. To minimize any chance of the honey bee DNA getting damaged and or drying up, all individuals were washed with distilled water before being placed in Eppendorf tubes containing 70% ethanol and 5% glycerin. After being assayed and appropriately labelled, these tubes were allowed to sit at room temperature for additional analysis. Information about the collection site including the date of collection, host, quantity of honey bees, and locality were also recorded at the time of sample collection. The collected specimens were morphologically identified using taxonomic keys outlined by^{21,22}. All collected specimens were morphologically recognized as *Apis mellifera*, *Apis cerana*, *Apis florea*, and *Apis dorsata*.

Isolation of bacteria

The bacteria associated with honey bee brood were isolated by adding 1 g of each comb piece in 9 mL of sterile water which was further diluted. The last dilution (100 µL) was cultured on the brain-heart infusion (BHI) agar media supplemented with thiamine hydrochloride (0.1 g/L) (DEVI et al., 2024). The plates were incubated overnight at 37 °C. The bacterial isolates were sub cultured by streak method and the purified colonies were stored in 20% glycerol stock at -20 °C for further characterization.

Biochemical characterization

To identify the bacterial isolates, biochemical tests were performed on freshly grown bacterial isolates.

Oxidase test

A drop of 10 µL of N, N-dimethyl-p-phenylenediamine, a reagent solution was poured on the filter paper and the bacterial colony was picked with the help of a loop and rubbed on the spot²³. The colony forming a blue color within 10 s was considered oxidase-positive.

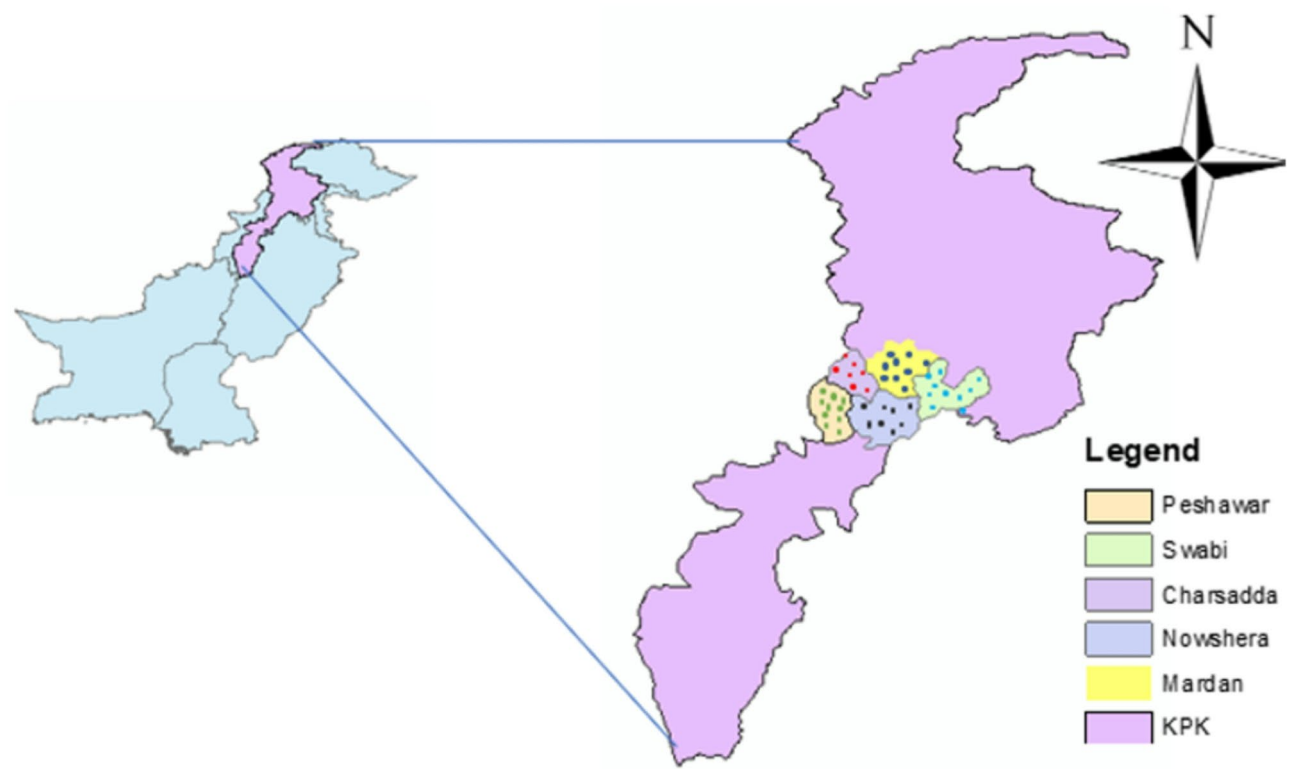


Fig. 1. Geographical map of the study districts showing the collection sites as colored dots.

| | Specie name | Host | Collection region | Accession number |
|-----------|--|---|-------------------|--|
| Pathogens | <i>Bacillus</i> spp <i>Bacillus Cerus</i> | <i>Apis melifera</i> <i>Apis Dorsata</i> <i>Apis melifera</i> | Mardan | OP901708, OP901717 OP901709 OP901713 |
| | <i>Bacillus</i> spp | <i>Apis melifera</i> <i>Apis Dorsata</i> <i>Apis florea</i> | Peshwara | OP901710 OP901711 |
| | <i>Bacillus</i> spp | <i>Apis melifera</i> <i>Apis Dorsata</i> <i>Apis florea</i> | Nowshera | OP901712 OP901714 |
| | <i>Bacillus</i> spp | <i>Apis melifera</i> <i>Apis Dorsata</i> <i>Apis florea</i> | Swabi | OP901715 OP901716 |

Table 1. Ticks and its associated pathogens sequence that submitted to gene bank.

Catalase test

A smear of bacterial colony was prepared on a glass slide. A drop (10 µL) of 3% hydrogen peroxide was added to the smear²⁴, leading to the formation of bubbles within 10 s confirmed the catalase producing isolates.

Sugar fermentation test

The nutrient broth (100 mL) containing 1% sugar (glucose, sucrose and lactose) was prepared to access the ability of bacterial isolates to ferment each sugar²⁵. Phenol red (800 mg) was added as an indicator. The isolates were inoculated in the broth media and incubated at 37 °C overnight. The conversion of red color of media to yellow indicated the positive sugar fermentation test.

Gram’s staining

The bacterial isolates were Gram stained according to the methodology described by Vincet²⁶. A smear of the isolate was made into a drop of water on the glass slide, which was then air-dried and heat-fixed. It was stained with a drop of crystal violet dye for 1 min and washed with distilled water for 2 s. A drop of iodine solution was added for 1 min followed by washing with decolorizer for 20 s. and then distilled water for 2 s. It was stained with a drop of safranin for 1 min and washed with distilled water for 2 s. The prepared slides were air-dried and observed under a microscope at 100 X magnification with oil emulsion. The isolates that retained violet color were Gram-positive while the isolates-stained pink were Gram-negative bacteria.

Endospore staining

Endospore staining for bacterial isolates was performed by Schaeffer Fulton method²⁷. A smear of bacterial colony was prepared into a drop of water on the glass slide. The smear was air dried and heat fixed. The area containing smear was covered with a piece of blotting paper. The blotting paper was saturated with 5% malachite green stain solution and kept on steam for 30 min. During the whole process, the blotting paper was kept moist with the stain. The slide was washed with distilled water and stained with a drop of safranin stain for 1 min followed by washing with distilled water. On air drying, the slide was observed under a microscope at 100 X magnification with emulsion oil. The endospores were stained green while the vegetative cells were stained pink.

DNA extraction

A modified CTAB method was used to extract genomic DNA from bacterial isolates²⁸. An appropriate amount of freshly grown culture on agar plates was taken into an Eppendorf containing pre-warmed 500 µL lysis buffer (2% CTAB; 6 M NaCl; 1 M Tris-HCl pH 8.0; 0.5 M EDTA). 40 µL of 10% SDS and 2 µL mercaptoethanol were added into the suspension and kept at 95 °C for 2 h. The extracted DNA was purified by layer separation technique with the addition of chloroform: isoamyl alcohol (24: 1) into suspension followed by centrifugation at 10,000 rpm for 10 min. The upper aqueous layer was removed into a clean Eppendorf. The DNA was precipitated by the addition of ice-chilled isopropanol (500 µL) into the collected aqueous layer. The supernatant was discarded and the pellet was washed with 70% ethanol, blot dried, and re-suspended in low TE buffer (50 µL) (0.1 mM EDTA, pH 8.0; 10 mM Tris-HCl). To dissolve the pellet completely in the buffer, it was incubated for half an hr. at 60 °C followed by storage at – 20 °C. 1% agarose gel was prepared to run extracted DNA which was then visualized under trans UV.

16 S rRNA gene amplification

A forward primer (5'- CCTAYGGGRBGCASCAG – 3') and a reverse primer (5'- GGACTACNNGGGTATCTAAT – 3')²⁹, were used to amplify the 468 bp gene of 16 S rRNA where bacterial DNA was used as a template. A reaction mixture of 25 µL was prepared by mixing master mix (12.5 µL) (Abclonal, USA), each primer (2 µL), PCR water (3.5 µL), and DNA (5 µL). The thermocycler was adjusted for 35 cycles with the conditions comprising of initial denaturation (95 °C for 10 min), denaturation (95 °C for 30 s), annealing (57 °C for 30 s), extension (72 °C for 90 s) and final extension (72 °C for 10 min). On completion, the amplified DNA was run on 1.5% agarose gel along with a DNA ladder (100 bp) for confirmation of the required product size.

DNA sequencing and phylogenetic analysis

Amplified PCR products were sequenced by a commercial service provider (First Base, Malaysia). The obtained DNA sequences underwent initial trimming to eliminate primer-contaminated regions and any misread nucleotides at the start and end of the sequence were also removed using FinchTV (version 1.4.0). All sequences were submitted to NCBI's GenBank and were assigned accession numbers: OP901708, OP901709, OP901710, OP901711, OP901712, OP901714, OP901715, OP901716, and OP901717 (*Bacillus* species), and OP901713 (*Bacillus cerus*) accession number in Table (1). Similar sequences for both *Bacillus* spp. and *Bacillus cerus*. were retrieved using the Basic Local Alignment Search Tool (BLAST) from NCBI. Subsequently, all the sequences were aligned using the ClustalW multiple sequence alignment algorithm in BioEdit (version 7.2.5). The aligned sequences were imported into MEGA X (version 10.2.6). A model selection test was conducted for all sequences, using MEGA's integrated model selection tool. The best-fit model was chosen based on Bayesian Information Criteria (BIC) and Akaike Information Criteria (AIC) values, with the model exhibiting the lowest BIC and AIC considered as the “best-fit” substitution model. Phylogenetic trees were constructed using the Maximum Likelihood algorithm in MEGA X with 1000 bootstraps.

Statistical analysis

All data related to tick infestation were inserted into MS Excel spreadsheet (version 2108). The data were then analyzed in Excel to calculate the total prevalence.

Results

The table and Fig. 2, provides information about the collection and molecular screening of honey bee species across four districts: Mardan, Peshawar, Nowshera, and Swabi. The data shows the number of honey bees collected and molecularly screened from each district and species. In total, 460 honey bees were collected, with 270 being screened for pathogens. The pathogen detection results indicate that *Bacillus* species were detected in bees from all species across the districts. The number of pathogen-positive samples varied from district to district and from species to species. In Mardan, the majority of samples screened were from *Apis mellifera* (31 samples), with *Bacillus* species detected in 9, and *Apis dorsata* (24 samples), with *Bacillus* species detected in 6. Other species, such as *Apis florea* and *Apis cerana*, did not show pathogen presence. In Peshawar, similar trends were observed, with *Bacillus* species found in 6 samples from *Apis mellifera* (29 screened), and 4 samples from *Apis dorsata* (19 screened). *Apis florea* also showed *Bacillus* species presence in 4 samples out of 16 screened. In Nowshera, *Bacillus* species were found in *Apis mellifera* (18 out of 28 screened), *Apis dorsata* (17 out of 18 screened), and *Apis florea* (2 out of 11 screened). However, *Apis cerana* showed no pathogen presence. In Swabi, *Bacillus* species were found in *Apis mellifera* (16 out of 21 screened), *Apis dorsata* (15 out of 19 screened), and *Apis florea* (2 out of 12 screened). No pathogen was detected in *Apis cerana* detailed information in Table (2). The total number of pathogen detections across all districts was 52, with the majority of detections being in *Apis mellifera* and *Apis dorsata*. This information could be valuable for further studies on the distribution of pathogens in different honey bee species across various regions. *Bacillus* species appear to be a common pathogen in the screened populations, but it is important to consider the role of other potential pathogens or environmental factors influencing the pathogen prevalence.

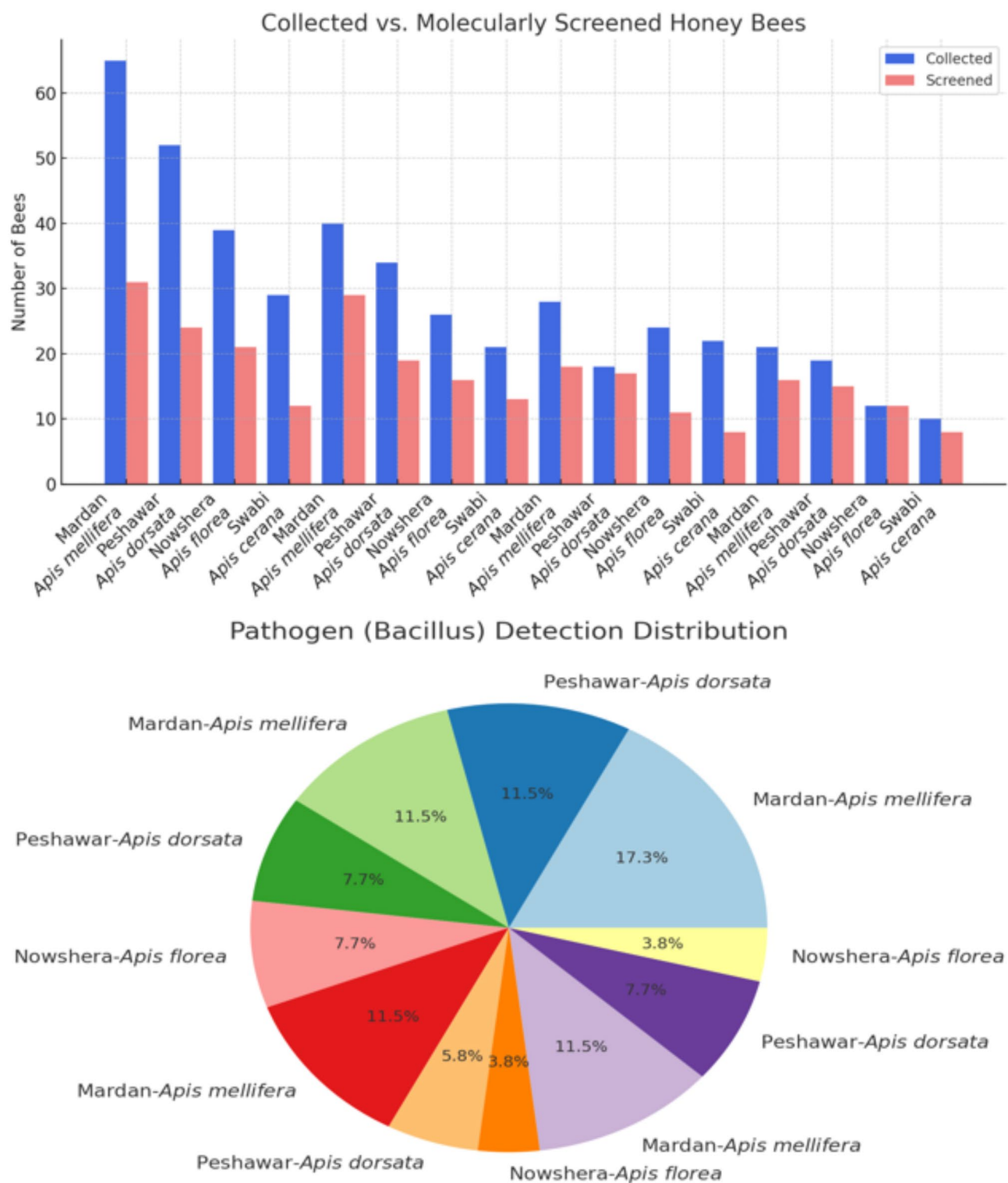


Fig. 2. Prevalence and molecular screened honey bee.

| District | Honey Bee species | Collected Honey Bee | Molecularly screened honey Bee | Pathogen Detection | Total |
|----------|-----------------------|---------------------|--------------------------------|-------------------------|-------|
| Mardan | <i>Apis mellifera</i> | 65 | 31 | <i>Bacillus species</i> | 9 |
| | <i>Apis Dorsata</i> | 52 | 24 | <i>Bacillus species</i> | 6 |
| | <i>Apis florea</i> | 39 | 21 | - | 0 |
| | <i>Apis Cerana</i> | 29 | 12 | - | 0 |
| Peshwara | <i>Apis mellifera</i> | 40 | 29 | <i>Bacillus species</i> | 6 |
| | <i>Apis Dorsata</i> | 34 | 19 | <i>Bacillus species</i> | 4 |
| | <i>Apis florea</i> | 26 | 16 | <i>Bacillus species</i> | 4 |
| | <i>Apis Cerana</i> | 21 | 13 | - | 0 |
| Nowshera | <i>Apis mellifera</i> | 28 | 18 | <i>Bacillus species</i> | 6 |
| | <i>Apis Dorsata</i> | 18 | 17 | <i>Bacillus species</i> | 3 |
| | <i>Apis florea</i> | 24 | 11 | <i>Bacillus species</i> | 2 |
| | <i>Apis Cerana</i> | 22 | 8 | - | 0 |
| Swabi | <i>Apis mellifera</i> | 21 | 16 | <i>Bacillus species</i> | 6 |
| | <i>Apis Dorsata</i> | 19 | 15 | <i>Bacillus species</i> | 4 |
| | <i>Apis florea</i> | 12 | 12 | <i>Bacillus species</i> | 2 |
| | <i>Apis Cerana</i> | 10 | 8 | - | 0 |
| | Total | 460 | 270 | | 52 |

Table 2. Honey bee and pathogen detection.

The Fig. 3, represents the distribution of *Bacillus* species across different honey bee species in various districts. The bar chart uses distinct colors to differentiate between *Apis mellifera*, *Apis dorsata*, *Apis florea*, and *Apis cerana*, making it easier to compare pathogen detection rates across districts. *Apis mellifera* shows the highest pathogen detection in all districts, with the highest occurrence in Mardan. This may be due to its managed nature, increased exposure to external environments, or susceptibility to bacterial infections. *Apis dorsata*, a wild honey bee species, also has notable detections, particularly in Mardan and Swabi, suggesting environmental exposure. *Apis florea* has fewer detections, with cases reported in Peshawar, Nowshera, and Swabi, while *Apis cerana* shows no detection of *Bacillus* species across all districts. The absence of the pathogen in *Apis cerana* might indicate possible resistance or a lower exposure to contaminated foraging sources.

Sequence similarity and BLAST analysis

In total 270 honey bee were used to extract DNA, and at least 52 honey bee produced positive amplicons for the 12 S RNA gene (468 bp), 20 sequences were acquired. The *Bacillus* species 16 S rDNA amplicons showed a 99–100% similarity range for 16 S rDNA after repeated sequence alignment. Compared to the BLAST analysis's sequences, the 16 S RNA partial sequence displayed a 99.15% or more identity with the *Bacillus* species partial

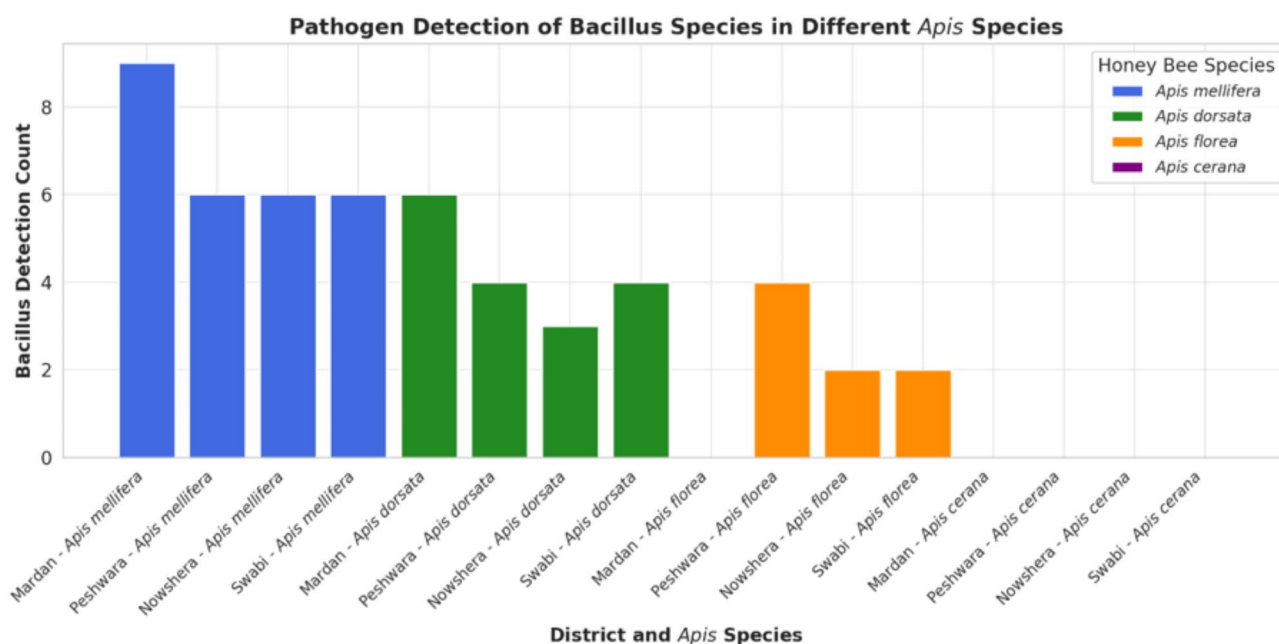


Fig. 3. Prevalence of pathogen in different honey bee species.

16 S sequence from India (PQ045992, PQ045996), *Bacillus Cereus* (OP901713) showed 99–100% identity with *Bacillus Cereus* that collected reported in Pakistan (MT421928).

Phylogenetic analysis

The evolutionary tree derived from the partial 16 S partial ribosomal DNA showed that the current study's haplotype were clustered with the 16 S sequence of *Bacillus* species previously reported from and India (PQ045992, PQ045996). All sequences reported from this study were grouped together with same number of nucleotide substitutions in the same clade. These haplotypes were genetically distinct from one another due to the similar amount of nucleotide changes, which was most likely caused by their shared origin in the same nation and infection of the same host. A number of *Bacillus*. sequences were included in the phylogenetic tree as references, including *Bacillus Paranthracis* incomplete 16 S rDNA sequence, which was reported from China (KF583644) and used as outgroup (Fig. 4).

Gram positive, rod-shaped bacterial species are depicted as follows in Fig. 5: (A) *Bacillus mycoides* and (B) *Bacillus cereus*. These microorganisms are related to each other, but have different shapes and biological functions. *Bacillus mycoides* is easily recognizable by its characteristic filamentous, spreading colonies, which is a unique means of differing him from other *Bacillus* species. On the other hand, *Bacillus cereus* has a notoriety as an opportunistic pathogen which is associated with food infection and toxin production. Despite their similar Gram-positive rod structures, their ecological roles and pathogenic potential vary significantly, making them intriguing subjects for microbiological studies.

The images in Fig. 6 display the significant green-colored spores from both *Bacillus mycoides* (A) and *Bacillus cereus* (B) which show the powerful resistance of Gram-positive bacteria. The formation of spore's functions as a survival mechanism because these microorganisms can survive harsh environmental conditions such as heat along with desiccation and chemical pressures. The green spores become visible by special staining techniques which demonstrates the dipicolinic acid-rich protective layers of the spores. Both *Bacillus mycoides* features spreading growth but *Bacillus cereus* has dangerous properties through its ability to produce toxins. Studying how these microorganisms create spores reveals important ecological and pathogenic information about them.

The bacterial isolates' morphological, biochemical, and Gramme staining properties are compiled in Table 3. The bacteria that were isolated were all rod-shaped and Gram-positive. They also tested positive for oxidase and catalase, which shows that they can use oxygen and break down hydrogen peroxide. Furthermore, all isolates showed their metabolic plasticity by fermenting lactose, glucose, and sucrose. Although more molecular identification is required for accurate classification, the consistency of the biochemical profile's points to a possible association with a particular bacterial genus.

Discussion

The Important information gained by the isolation and characterization of *Bacillus* species on the intricate microbial interactions with honey bee broods. The significance of other bacterial species in honey bee health is still little understood, despite the fact that *Paenibacillus larvae* and *Melissococcus plutonius* are well-established etiological agents of American foulbrood (AFB) and European foulbrood (EFB), respectively^{30,31}. *Bacillus cereus* and *Bacillus mycoides* were found in infected *Apis mellifera* broods for the first time in Pakistan by our investigation, indicating their possible function as secondary pathogens or opportunistic invaders that worsen colony health.

The phylogenetic analysis revealed a close evolutionary relationship between *Bacillus* species and *Bacillus cereus*. This clustering is consistent with the well-established observation that spore formation is a conserved trait within the genus *Bacillus*. Spore formation serves as a critical survival mechanism, allowing these bacteria to withstand harsh environmental conditions and persist in diverse ecological niches, including the honey bee brood environment. The shared capacity for spore formation likely contributes to the genetic similarity observed in our study, supporting the hypothesis that these organisms have evolved similar adaptive strategies. This finding aligns with previous reports on *B. cereus* in honey bee colonies displaying illness symptoms^{32,33}, and provides a plausible explanation for the close phylogenetic relationship noted between the isolates in our analysis. A spore-forming bacteria that is extremely durable and able to tolerate harsh environmental conditions, *B. cereus* is well-known for its link to human foodborne diseases³⁴. Concerns regarding its possible effects on honey bee larvae, namely its capacity to reduce immunity and survival, are raised by its capacity to create cytotoxins, hemolysins, and enterotoxins³⁵. *B. cereus* has been found in unhealthy honey bee broods in both North American and European studies, supporting its possible involvement in the loss of colony health³².

Important virulence genes, such as the cry gene, which has been linked to toxicity in insect hosts, were found in the *B. cereus* isolates in our investigation after molecular characterization³². These results imply that *B. cereus* strains recovered from honey bee larvae may share harmful characteristics with strains found in other hosts³². The bacterium may persist and play a role in the dynamics of honey bee disease, as evidenced by its exceptional adaptability to nutrient-rich but limited hive habitats. The idea that *B. cereus* may work in concert with primary pathogens is supported by earlier research conducted in China and the US, which similarly found toxin-producing strains of the bacteria in honey bee colonies¹⁷.

Our investigation also found *B. mycoides* in the broods of diseased honey bees. This close relative of *B. cereus* is distinguished by its soil-dwelling habit and unique filamentous colony shape^{36,37}. Its capacity to generate bioactive chemicals raises the possibility that it plays a part in microbial dysbiosis within hives, even though its pathogenicity in honey bees is still unknown³⁷. Concerns regarding *B. mycoides'* opportunistic behaviour and potential role in brood infections have been raised by reports from Sweden and Canada that similarly documented the parasite in ill honey bee colonies³⁸.

A larger and more complex microbial ecology within honey bee colonies is shown by the presence of *B. cereus* and *B. mycoides* in diseased broods. According to this research, microbial co-infections with bacteria like



Fig. 4. A phylogenetic tree showing evolutionary relatedness of the bacteria species associated with honey bee brood disease based on 16 S rRNA gene homology.

Bacillus, fungi, and viruses can have a big impact on colony health³⁹. Secondary bacterial invaders can worsen primary infections, weakening colony defenses and increasing larval mortality, according to research from Brazil and Italy⁴⁰. These results highlight how crucial it is to examine honey bee illnesses in the context of microbial ecology as a whole.

The prevalence and aggressiveness of *Bacillus* infections may also be influenced by environmental factors such as pesticide exposure, climate change, and contemporary beekeeping techniques⁴⁰. *B. cereus* and *B. mycoides*

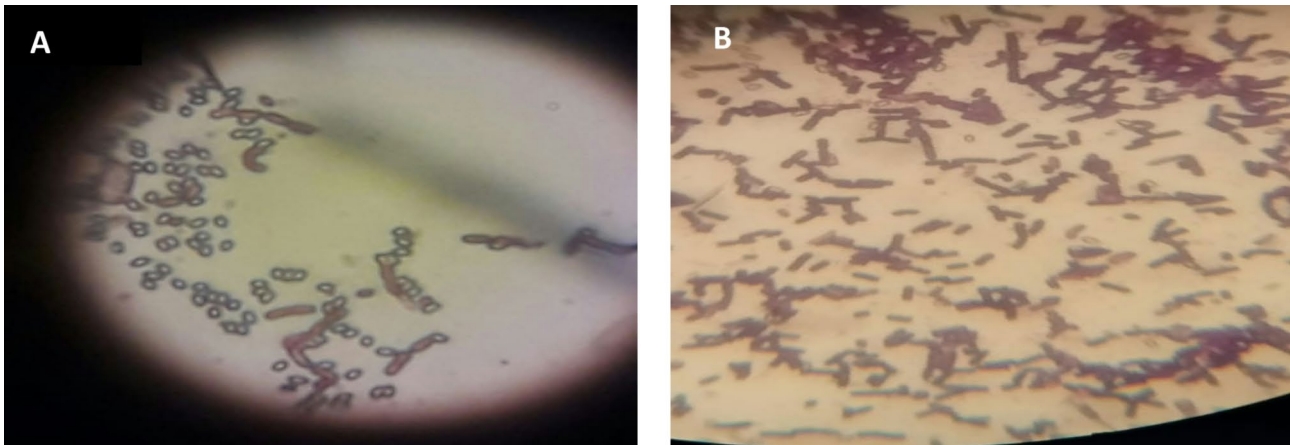


Fig. 5. (A) Gram-positive Rod-shaped *Bacillus mycoides* (B) Gram-positive Rod-shaped *Bacillus cereus*.

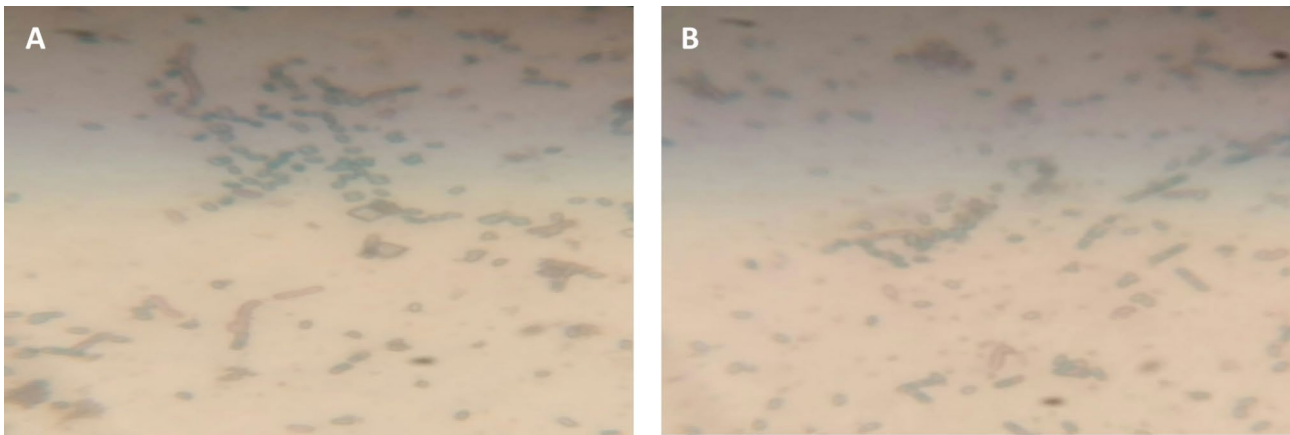


Fig. 6. (A) The green-colored spores of *Bacillus mycoides* (B) The green-colored spores of *Bacillus cereus*.

| Bacterial Isolate | Gram Staining | Morphology | Catalase Test | Oxidase Test | Sucrose Fermentation | Glucose Fermentation | Lactose fermentation |
|-------------------|---------------|------------|---------------|--------------|----------------------|----------------------|----------------------|
| HB1 | +ve | Rods | +ve | +ve | +ve | +ve | +ve |
| HB2 | +ve | Rods | +ve | +ve | +ve | +ve | +ve |
| HB4 | +ve | Rods | +ve | +ve | +ve | +ve | +ve |
| HB5 | +ve | Rods | +ve | +ve | +ve | +ve | +ve |
| HB6 | +ve | Rods | +ve | +ve | +ve | +ve | +ve |
| HB7 | +ve | Rods | +ve | +ve | +ve | +ve | +ve |
| HB8 | +ve | Rods | +ve | +ve | +ve | +ve | +ve |
| HB9 | +ve | Rods | +ve | +ve | +ve | +ve | +ve |
| HB13 | +ve | Rods | +ve | +ve | +ve | +ve | +ve |
| HB14 | +ve | Rods | +ve | +ve | +ve | +ve | +ve |

Table 3. Gram’s staining, morphology and biochemical tests of bacterial isolate.

spores are so resilient, there are worries that they may survive in pollen, hive materials, and even honey bee digestive tracts⁴¹. Expanding disease surveillance systems to include a greater spectrum of bacterial pathogens is essential given the ecological and economic importance of beekeeping in Pakistan. Deeper understanding of the relationships between *Bacillus* species and other microbial communities within *Apis mellifera* colonies can be obtained through transcriptome research, metagenomic investigations, and advanced molecular diagnostic methods. Additionally, by encouraging good bacterial populations, probiotic-based strategies present intriguing substitutes for disease

management. According to recent research from Germany and France, probiotic administration can improve honey bee gut microbiota and increase colony resistance to infections^{42,43}.

Conclusion

Our study provides the first molecular and phylogenetic evidence of *Bacillus* species, particularly *B. cereus* and *B. mycoides*, as emerging bacterial threats to honey bee brood health in Khyber Pakhtunkhwa, Pakistan. The detection of key virulence genes, including the *cry* gene, underscores the potential pathogenicity of these bacteria, which may contribute to larval mortality through cytotoxins, hemolysins, and enterotoxins. The close evolutionary relationship between *Bacillus* species highlights their adaptive strategies, raising concerns about microbial dysbiosis and weakened immunity in developing larvae. Given the global reports linking *Bacillus* infections to honey bee population declines, our findings emphasize the urgent need for further research into their pathogenic mechanisms and potential mitigation strategies. This study not only expands our understanding of bacterial infections in honey bee brood but also underscores a previously overlooked risk to apiculture and pollination services in the region.

Data availability

Sequences are submitted to GenBank with the accession numbers to the *Bacillus* species given by GenBank are as followed: OP901708, to OP901717, *Bacillus Cereus* HB14 OP901713. The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

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Declarations

Competing interests

The authors declare no competing interests.

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